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PATENT SPECIFICATION

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763,055

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COMPLETE SPECIFICATION

Improvements in and relating to the Production of Ascorbic Acid

We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, of 1, Tilney Street, London, W.1, a British Corporation, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

It is known that L-ascorbic acid is produced in vegetable and most animal organisms from hexose sugars. Evidence has also been found that in the case of glucose and galactose only, the conversion comprises a series of reactions the last stage of which is the oxidation, catalysed by certain enzymes, of the γ-lactone of an acid derived from sugar, i.e. from D-glucose, L-gulono-γ-lactone or from D-galactose, L-galactono-γ-lactone.

Our present invention is based on our discovery that the above mentioned lactones of L-gulonic and L-galactonic acid (and the methyl, ethyl and propyl esters and amides thereof) can in fact be oxidised to L-ascorbic acid outside the living organism in the presence of oxygen and at a pH of the extraction medium between 6.0 and 8.5 by means of a group of enzymes obtained as an extract from both animal and vegetable sources said group being one which transfers electrons from sugar lactones to molecular oxygen and includes a dehydrogenase and cytochrome oxidase or similar enzymes, but the extract not including substantial proportions of enzymes which oxidize ascorbic acid. Such groups of enzymes containing a dehydrogenase and cytochrome oxidase are present in the mitochondria occurring in seeds and animal tissues and similar enzyme systems are believed to be contained in the mitochondria of algae, yeasts and other micro-organisms. The enzymes may be obtained either from animal or vegetable tissues but since vegetable seeds also develop enzymes which oxidise ascorbic acid, in the case of vegetable seeds the germination time before extraction should not be so long that substantial quantities of these oxidising enzymes are present. It is found that they are not developed in the early stages of germination, so that with pea seeds, for example, a germination period of 12 to 48 hours or even more is suitable The extraction may be effected with a preferably cooled solution of an organic non-electrolyte having an osmotic pressure sufficient to prevent disintegration of the mitochondria, e.g. sucrose, mannitol, glycerol or urea, and also containing sufficient phosphate or arsenate to give it a pH of between 6.0 and 8.5, together with a trace of magnesium salt e.g., sulphate. It has been found in this connection that the optimum concentration of sucrose is 0.4 M with plant tissues but 0.25 M with animal tissues, while that of the phosphate or arsenate is 0.1 M. The magnesium salt may be used in a concentration of 4×10^{-3} M. We have found that pea seeds are an eminently suitable source of the enzyme in the case of plant tissues. The pH of the extraction medium has an important bearing on the oxidation of the lactone. No conversion occurs below 6.0 or above 8.5 and the optimum pH is about 7.4—7.5. The temperature co-efficient of the reaction is between 2 and 3 and 37° C. appears to be the optimum temperature for obtaining a reasonably rapid conversion without destruction of enzyme.

The reaction depends on the presence of oxygen, hence the reaction is conveniently carried out under aerobic conditions, although the oxygen pressure in the air may be reduced to 2.5 per cent. without an adverse effect on the rate of conversion. However, where large volumes of solution are used, a system of aeration may be necessary.

By way of example, the invention may be carried out in detail as follows:—

EXAMPLE 1.

30 gms pea seeds are soaked in water for germination periods of 12—48 hours and extracted after crushing with 40 ml of solution of 0.4 M sucrose at 0° C. containing 0.1 M phosphate pH 7.5 and 0.1 per cent. of mag-

[*Price 3s. 0d.*]

nesium sulphate. The extract is centrifuged to remove starch and cell debris. The supernatant solution (Extract A) at this stage may be purified if desired (though this is not essential) by further centrifugation at 10,000 g for 20 minutes at +1° C. The elements of the cell, in which the activity resides, are thrown down as a fine precipitate. This precipitate is suspended in the sucrose/PO_4 Mg solution (Extract B).

The lactone which may be the γ-lactone of either L-galactonic or L-gulonic acid is added in concentrations varying between 0.5—2.0 mg per ml of extract, which may be either A or B. The extract with lactone added is incubated at 37° C. for 4—6 hours after which time the maximum formation of L-ascorbic acid is obtained. This may be isolated by any suitable chemical or physical technique, e.g., ion exchange.

The percentage conversion of the lactone to ascorbic acid is relatively greater with the lower concentrations of lactone, and this is almost certainly due to the concurrent conversion of the lactone to the free acid, a reaction which proceeds competitively with the desired oxidation of the γ-lactone to L-ascorbic acid. With concentrations of lactone of the order of 0.5 g—1.0 g ml of extract, the conversion is of the order of 40 per cent.

After a period of 4—5 hours, additional amounts of the γ-lactone may be added, and a further quantity of L-ascorbic acid obtained. In this way it is possible to continue to promote the synthesis still further.

Although from 12—48 hours has been given as the germination time in this Example, still longer times are permissible provided they are not so prolonged as to lead to the presence of substantial quantities of enzymes which oxidise ascorbic acid.

It is possible under the above conditions to convert not only the γ-lactone of galactonic or gulonic acid but also the esters, in particular the methyl, ethyl or propyl esters and amides thereof; the free acid itself is, however, not converted. In working with esters the quantity may be increased in proportion to the molecular weight, though the reaction also proceeds if the same quantities are used. In the case of amides, since simple amides have molecular weights of the same order as the lactones, the same quantities may be used.

EXAMPLE 2.

Rat liver (10 g) is homogenised in 0.25 M sucrose solution (100 ml.) and the extract centrifuged to remove cell debris. The supernatant solution which contains the particulate elements of the cell together with soluble enzymes is then centrifuged at 20,000 g for 1 hour at +1° C. The particulate elements in which the activity resides are thrown down. This precipitate is resuspended in 0.25 M sucrose/0.1 M phosphate buffer (pH 7.4). Other experimental conditions were similar to those for peas.

The γ-lactone of L-galactonic or L-gulonic acid is added in concentration varying from 0.1 to 2.0 mg./ml of extract. The extract with lactone is incubated at 37° C. for 1—2 hours, after which time the maximum formation of L-ascorbic acid is obtained.

Methyl, ethyl or propyl esters or amides may also be used as above mentioned in Example 1. The L-galactono-γ-lactone may itself be prepared from D-galacturonic acid or esters thereof by reduction by suitable chemical methods, e.g., by catalytic hydrogenation. D-galacturonic acid may be cheaply prepared by the enzymic degradation of pectic acid and other complex uronides of vegetable origin. (Ayres, Dingle, Phipps, Reid and Solomons, Nature, 1932, *170*, 834).

In addition to the converting the γ-lactones, esters and amides of L-galactonic and L-gulonic acid, the whole cell extract is also able to convert the esters and lactones of D-galacturonic acid and D-glucuronic acids. In this case, the whole cell extract also contains a reductase-like enzyme, that is a reducing enzyme, which catalyses the transfer of hydrogen or electrons from reduced tri-phosphopyridine nucleotide (co-enzyme II) to the derivatives of the uronic acid, thus producing the corresponding derivative of L-gulonic or L-galactonic acid *in situ*. These derivatives are then converted by the group of enzymes above specified. The quantities of derivatives of D-galacturonic and D-glucuronic acid added to the enzyme extract should be at least 5 mgs/ml of extract. The overall conversion is less efficient than of the γ-lactone, esters and amides of L-galactonic and L-gulonic acid and it is preferable therefore first to prepare the lactone on the above mentioned lines.

What we claim is:—

1. A process for the production of L-ascorbic acid from one or more of the γ-lactone, and methyl, ethyl and propyl esters and amides of L-gulonic or of L-galactonic acid, which comprises oxidising in the presence of oxygen one or a mixture of such substances by the action of a group of enzymes obtained in an extraction with a medium at a pH between 6.0 and 8.5 from animal or vegetable tissues, said group being one which transfers electrons from sugar lactones to molecular oxygen and includes a dehydrogenase enzyme and cytochrome oxidase, but the extract not including substantial proportions of enzymes which oxidise ascorbic acid.

2. A process as claimed in claim 1 in which the gamma lactones or esters of L-gulonic or L-galactonic acid are prepared *in situ* by the reduction of the corresponding derivatives of D-glucuronic and D-galacturonic acids catalysed by the action of an enzyme system in the whole cell extract.

3. A process as claimed in claim 1 or 2

wherein the said group of enzymes is obtained either from animal or vegetable tissues by extraction with a preferably cooled solution of an organic non-electrolyte having an osmotic pressure sufficient to prevent disintegration of the mitochondria and containing sufficient phosphate or arsenate to give a pH of between 6.0 and 8.5 together with a trace of magnesium salt.

4. A process as claimed in claim 3 wherein the organic non-electrolyte is either sucrose, mannitol, glycerol or urea.

5. A process as claimed in claim 3 or 4 wherein the organic non-electrolyte is sucrose at a concentration of 0.4 M with plant tissue and 0.25 M with animal tissue.

6. A process as claimed in claim 6 wherein the concentration of phosphate or arsenate is 0.1 M.

7. A process as claimed in any of claims 3 to 7 wherein the magnesium salt has a concentration of 4×10^{-3} M.

8. A process as claimed in any of claims 3 to 8 wherein the pH value is between 7.4 and 7.5.

9. A process as claimed in any of the preceding claims wherein the said group of enzymes is obtained by extraction from pea seeds after germination for a period of 12 to 48 hours.

10. A process as claimed in any of claims 1 to 8 in which the said group of enzymes is obtained from animal tissues by a period of centrifuging of sufficient intensity and length to precipitate the particulate elements in which activity resides.

11. A process as claimed in any of claims 3 to 10 wherein the temperature of the mixture of the substance and the enzyme extracts is approximately 37° centigrade.

12. A process as claimed in any of claims 3 to 11 wherein to provide for the presence of oxygen the reaction is carried out under arerobic conditions.

13. A process as claimed in claim 1 and as illustrated by the examples herein.

14. L-ascorbic acid obtained by the process according to any of the preceding claims.

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PROVISIONAL SPECIFICATION

Improvements in and relating to the Production of Ascorbic Acid

We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, a British Corporation, of 1, Tilney Street, London, W.1, do hereby declare this invention to be described in the following statement:—

It is known that L-ascorbic acid is produced in vegetable and most animal living organisms from hexose sugars. Evidence has also been found that in the case of glucose and galactose only, the conversion comprises a series of reactions the last stage of which is the oxidation, catalysed by certain enzymes, of the γ-lactone of the acid derived from the sugar, i.e. of L-gulono-γ-lactone or of L-galactono-γ-lactone.

Our present invention is based on our discovery that the above mentioned lactones can in fact be oxidised to L-ascorbic acid outside the living organism by means of enzymes obtained from vegetable sources; the enzymes in question are believed to be a certain dehydrogenase and cytochrome oxidase. Such enzymes are present in the mitochondria occurring in seeds and animal tissues and similar enzyme systems are believed to be contained in the mitochondria of algae, yeasts and other micro organisms. The enzymes may be obtained either from animal or vegetable tissues by extraction with a preferably cooled solution of an organic non-electrolyte having adequate osmotic pressure, e.g. sucrose, mannitol, glycerol or urea and also containing sufficient phosphate or arsenate to give it a pH of between 6.0 and 8.5, together with a trace of magnesium salt e.g. sulphate. It has been found in this connection that the optimum concentration of sucrose is 0.4 M while that of the phosphate or arsenate is 0.1 M. The magnesium salt may be used in a concentration of 4×10^{-3} M. We have found that pea seeds are an eminently suitable source of the enzyme. The pH of the extraction medium has an important bearing on the oxidation of the lactone. No conversion occurs below 6.0 or above 8.5 and the optimum pH is about 7.4—7.5. The temperature co-efficient of the reaction is between 2 and 3 and 37° C. appears to be the optimum temperature for obtaining a reasonably rapid conversion without destruction of enzyme.

The reaction depends on the presence of oxygen, hence the reaction is conveniently carried out in the presence of air, although the oxygen tension may be reduced to 2.5 per cent. without an adverse effect on the rate of conversion. However, where large volumes of solution are used, a system of aeration may be necessary.

By way of example, the invention may be carried out in detail as follows:—

30 gms pea seeds are soaked in water for periods of 12—48 hours and extracted after crushing with 40 ml. of an ice-solution of 0.4 M sucrose containing 0.1 M phosphate pH 7.5 and 0.1 per cent. of magnesium sulphate. The extract is centrifuged to remove starch and cell debris. The supernatant solution (Extract A) at this stage may be purified if de-

sired (though this is not essential) by further centrifugation at 10,000 g for 20 minutes at +1° C. The elements of the cell, in which the activity resides, are thrown down as a fine precipitate. This precipitate is suspended in the sucrose/PO_4 Mg solution (Extract B).

The lactone is added in concentrations varying between 0.5—2.0 mg per ml of extract, which may be either A or B. The extract with lactone added is incubated at 37° C. for 4—6 hours after which time the maximum formation of L-ascorbic acid is obtained. This may be isolated by any suitable chemical or physical technique, e.g. ion exchange.

Only the γ-lactone of galactonic acid, but not the free acid itself, is converted to L-ascorbic acid under these conditions. The percentage conversion of the lactone to ascorbic acid is relatively greater with the lower concentrations of lactone, and this is almost certainly due to the concurrent conversion of the lactone to the free acid, a reaction which proceeds competitively with the desired oxidation of the γ-lactone to L-ascorbic acid. With concentrations of lactone of the order of 0.5 g—1.0 g ml of extract, the conversion is of the order of 40 per cent.

After a period of 4—5 hours, additional amounts of the γ-lactone may be added, and a further quantity of L-ascorbic acid obtained. In this way it is possible to continue to promote the synthesis still further.

Although from 12—48 hours has been given as the germination time in this Example, still longer times are permissible.

The L-galactono-γ-lactone may itself be prepared from D-galacturonic acid or esters thereof by reduction by suitable chemical methods, e.g. by catalytic hydrogenation. D-galacturonic acid may be cheaply prepared by the enzymic degradation of pectic acid and other complex uronides of vegetable origin. (Ayres, Dingle, Phipps, Reid and Solomons, Nature, 1932, *170*, 834).

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